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13. ABSTRACT (Maximum 200 words) Maximum microvessel density (MMVD) in primary tumors is a sensitive prognostic indicator for many solid tumors, including breast. These data imply functional significance for the "hot spots" of microvessels which are quantitated in MMVD determinations. Since prognosis is determined by metastasis, functional significance of the "hot spots" in the process of metastasis is also implied. We hypothesize that the neovascular "hot spots" are the sites of metastasis and that gene expression in the tumor in the vicinity of the "hot spot" is important in the generation of the "hot spot" and in the process of metastasis. We are looking for differences in gene expression in the tumor in areas adjacent to "hot spots" when compared to other areas of the tumor. Our model system is FGF-1 transfected MCF-7 breast carcinoma cells growing as xenografts in nude mice. We have correlated metastasis with tumor size and are endeavoring to correlate MMVD with metastasis. We are developing methods of isolating RNA from microdissected tissue, amplifying it, and constructing cDNA libraries. When we have determined the "hottest spot" of microvessel density in the most metastatic tumors, we will microdissect areas of tumor adjacent to them and compare gene expression in areas not adjacent to hot spots.				
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FOREWORD

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Jonathan W. McLeskey 7/30/98
PI - Signature Date

TABLE OF CONTENTS

1.	FRONT COVER-----	1
2.	REPORT DOCUMENTATION PAGE-----	2
3.	FOREWORD -----	3
4.	TABLE OF CONTENTS-----	4
5.	INTRODUCTION-----	5
6.	BODY-----	7
7.	CONCLUSION-----	14
8.	REFERENCES-----	15
9.	APPENDIX-----	17
	List of abbreviations and acronyms-----	18
	Meeting abstracts since last report-----	19
	Publications since last report-----	19
	Manuscripts in preparation-----	19
	Personnel receiving pay from this negotiated effort-----	19

INTRODUCTION

Strong evidence has emerged linking maximal microvessel density with poor prognosis and metastasis in breast and other types of cancer. Neovessels which are counted in quantitation of maximal microvessel density are most often in the stroma of the tumor and form "hot spots" of high density. If blood vessel density determinations are not done by quantitation of microvessel density in hot spots, the correlation between prognosis and microvessel density is lost [reviewed in (1)]. Since the blood vessel density only in the area of the hot spot is a determinant of outcome, a functional significance for the microenvironment of the hot spot in the process of metastasis is suggested.

Metastasis is a multistep process involving invasion of the tumor through basement membrane, intravasation into blood or lymphatic vessels, attachment to endothelium at distant sites, extravasation, and growth of metastatic cells (2). We believe that microvascular hot spots are the sites of intravasation of tumor cells and that the acquisition of the ability to generate hot spots of high microvessel density increases the tumor's ability to disseminate throughout the body. The acquisition of the ability to generate "hotter spots" may result from clonal variation which arises within the tumor. This line of reasoning implies that unknown factors which are not expressed elsewhere in the tumor are responsible for the development of the hot spot and that gene expression in the area of the hot spot is an important determinant of a tumor's ability to intravasate. We hypothesize that gene expression in the area of the hot spot is different from elsewhere in the tumor and that genes expressed in the microenvironment of the hot spot are important in the process of intravasation as well as in the formation of the hot spot. This proposal has the purpose of identifying known or unknown genes which are differentially expressed in the area of the tumor adjacent to the area of maximal microvessel density. Once such genes are identified, their specific function in the process of tumor cell dissemination can be determined and therapies which address this process can be developed.

We have developed an experimental tumor system which utilizes FGF-1 transfected MCF-7 cells. These cells are metastatic to lymph nodes and lungs and express bacterial *lacZ*, enabling their colorimetric detection (3-5). Tumors formed by the transfectants exhibit hot spots of microvasculature; preliminary results with separate experiments using this tumor system correlate maximal microvessel density with tumor size (not shown) and tumor size with the degree of metastasis [(4,6,7) and this report]. Thus, it is likely that maximal microvessel density and degree of metastasis can be positively correlated within a single experiment. We proposed to utilize this system to examine multiple tumors, determine the "hottest" spot of microvessel density in each tumor and accurately correlate that microvessel density with the degree of metastasis detected in

McLeskey, Sandra W.
DAMD17-94-J4173

lymph nodes and lungs. These determinations will accurately identify specific areas of each tumor which express high levels of transcripts of genes involved in the development of the hot spot. We will microdissect these areas of the tumor and compare their gene expression with areas of the tumor not involved in hot spot generation using amplified fragment length polymorphism (AFLP). This scheme and other controls incorporated into the proposed AFLP protocol ensures that the differences between the mRNA populations compared are maximal and that the project will identify transcripts differentially expressed in the area of the hot spot and not elsewhere in the tumor. We will verify candidate transcripts' differential expression in tumor areas adjacent to hot spots utilizing dot blots and library probes made from the microdissected tissue, followed by *in situ* hybridization. Only candidate transcripts which exhibit selective expression in tumor microenvironments associated with hot spots and not elsewhere in the tumor will be pursued to obtain a full-length cDNA. Experiments would follow which examine function of differentially expressed genes in the development of hot spots or dissemination of tumor cells. Thus, this proposal would be the beginning of a mechanistic investigation into one of the steps of metastasis --- formation of microvessel hot spots and tumor cell intravasation. Elucidation of the mechanism for this step could provide knowledge which would target therapeutic interventions toward preventing it.

BODY

Aim 1. We will identify critical microenvironments in the tumors produced by FGF-1 transfected MCF-7 cells in nude mice by sensitively and accurately correlating the degree of metastasis in lungs and lymph nodes with maximal microvessel density in the hottest spot in each tumor.

Cell lines. ML-20 cells, which have been previously described (3,4), are a clonal line of wild-type MCF-7 cells transfected with an expression vector containing the cDNA for bacterial *lacZ*. We find their *in vivo* behavior to be indistinguishable from wild-type MCF-7 cells. Clone 18 cells are a clonal line of ML-20 cells transfected with an expression vector encoding FGF-1. Clone 18 cells exhibit estrogen-independent and tamoxifen-resistant growth in ovariectomized nude mice and form very large tumors when mice are treated with estrogen (5). In contrast to the parental ML-20 cells or wild-type MCF-7 cells, these tumors are reliably metastatic to lymph nodes and lungs. By virtue of the *lacZ* transfection, micrometastases can be sensitively detected in distant organs. Clone 18 cells were used for tumor production (below) for this project.

Tumor production, harvest, and processing. Ten million Clone 18 cells/0.2 ml were injected into the mammary fat pads of 30 ovariectomized nude mice at one site (right inguinal mammary fat pad) as previously described (3-5). Mice were treated with 60-day sustained release pellets containing 17 β -estradiol (0.72 mg, Innovative Research, Sarasota, FL). Tumors were measured in three directions biweekly. Tumors were harvested with adjacent stroma by cutting through the skin and peritoneum in a circular incision circumscribing the tumor, weighed, and immediately frozen in embedding medium (Tissue-Tek OCT, Sakura, Torrance, CA) by immersion in liquid nitrogen. Embedded tumors were stored at -70° prior to sectioning.

Concurrently with tumor harvesting, lungs of all animals were harvested and subjected to X-gal staining as previously described (3,4). Harvested, stained lungs were used to quantify metastases, below.

Detection and quantification of pulmonary metastases: Following harvesting and brief fixation in 2% formaldehyde/0.02% glutaraldehyde in phosphate buffered saline, lungs were incubated overnight with X-gal staining solution at 4° (4), followed by 48 hours fixation in 10% buffered formalin. Lungs were examined with a dissecting microscope (Olympus AH-2) with attached videocamera. Images of representative areas of lungs were acquired and blue-staining micrometastases quantified using the Optimas image analysis software. Areas of lung examined were also quantified using a standard grid and the Optimas software, and micrometastases/unit area

was calculated. As has previously been shown with FGF-4 transfected MCF-7 cells (4,6) as well as the Clone 18 cells (7), density of micrometastases in this experiment correlated with tumor weight (Figure 1).

Topographical analysis of tumors:

Before freezing, tumors were cut into approximately 3-4 mm thick slices as depicted in Figure 2. Each slice was named as to its location in the tumor and embedded in a separate cassette. This scheme enables us to identify the location of particular slices in the original tumor. In that way, topographical locations of particular microvessel "hot spots" can be localized.

Immunohistochemistry for PECAM-

1. Immunohistochemistry for murine PECAM-1 was accomplished using the rat monoclonal antibody Mec 13.3 (8) [Pharmingen (01951D)]. Following brief fixation in 70% ethanol, endogenous peroxidases were quenched in 0.3% H_2O_2 in methanol for 30 minutes. Blocking solution (2% normal rabbit serum, 5% bovine serum albumin (Sigma #A7888) in PBS was applied for 1 hour. Primary rat anti-murine PECAM-1 antibody was applied for 2 hours at room temperature in blocking solution at a concentration of 5 $\mu\text{g}/\text{ml}$. After PBS washes, secondary rabbit anti rat antibody coupled to biotin (Vector #BA4001) was applied for 1 hour. Slides were washed three times in PBS and ABC reagent coupled to peroxidase (Vector Elite kit #PK6001) was applied for one hour. Following three PBS washes, slides were incubated with 0.05% diaminobenzidine and 0.01% H_2O_2 for 15 minutes. Slides were then briefly counterstained with Harris acidified hematoxylin, washed, dehydrated in graded alcohols and coverslipped from xylenes (9).

Vessel quantification and identification of hot spots: Frozen slices were sectioned serially onto uncoated slides and representative slides from each slice were immunostained with the antibody to PECAM-1 for microvessel hot spot quantification. Since coverslipped slides cannot be subjected to microdissection, the slides selected for this staining were from widely spaced areas (about every 0.25 mm) of the tumor. Unstained intervening sections were preserved at -70° . Areas of high microvessel density were identified in the stained slides under low power and quantified at 100X

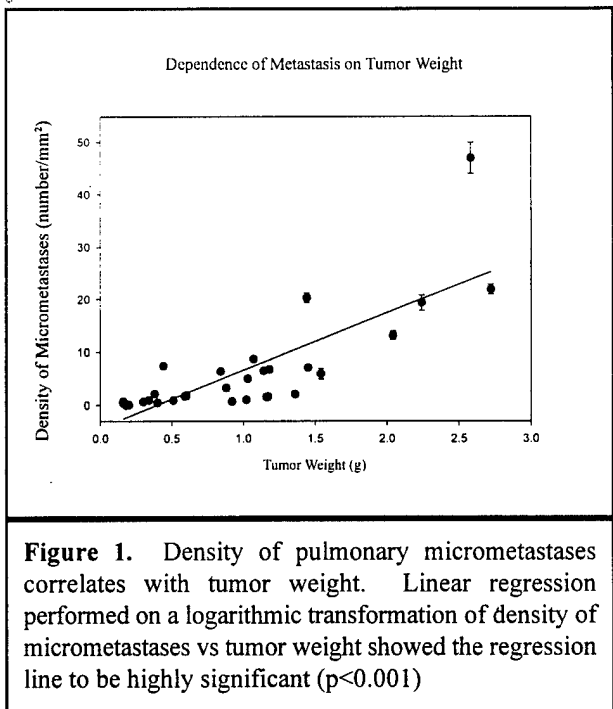
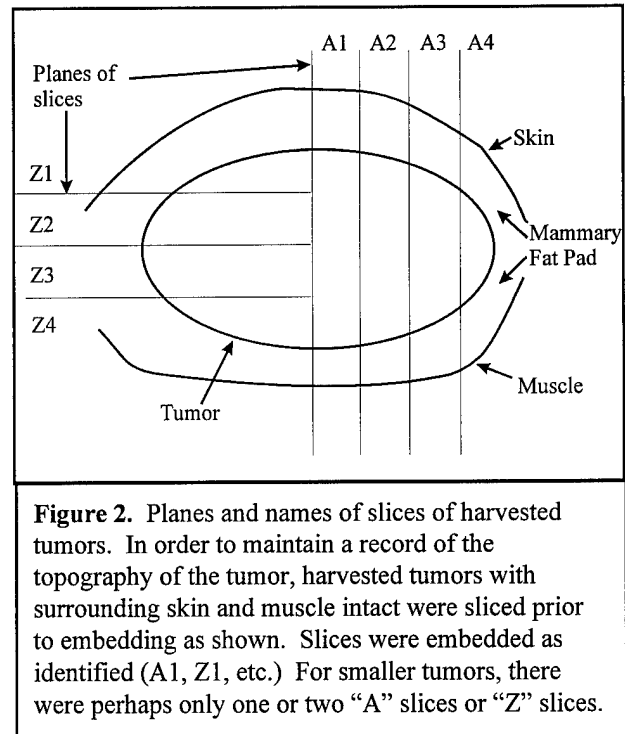


Figure 1. Density of pulmonary micrometastases correlates with tumor weight. Linear regression performed on a logarithmic transformation of density of micrometastases vs tumor weight showed the regression line to be highly significant ($p < 0.001$)

magnification by use of a Chalkley graticule (10) (Graticules Limited, Tonbridge Kent, U.K.). This instrument inserts into the eyepiece of the microscope and produces a field divided into quartiles with randomly placed dots in each quartile. The operator places one of the dots over one of the features of interest (in this case, a microvessel) and counts the number of other dots which are also positioned over the features of interest in that field. The number of dots coinciding with features of interest (microvessels) is the "Chalkley score". There are a total of 25 dots in the whole field, so the lowest possible score is 1 and the highest possible score is 25. We are in the process of scoring the representative PECAM-1 stained sections from all the tumors. We have scored most of the 30 tumors for the A1 slice and many of the tumors from other slices. In addition to identifying the section from each of the most metastatic tumors for the "hottest spot" (below), we will be analyzing these data to see whether a particular area of the tumor has "hotter" spots of microvessels.



Correlation of hot spots and degree of metastasis: This analysis is underway.

Aim 2. We will analyze differential gene expression in tumor cells in the area of hot spots by microdissection followed by differential display PCR and *in situ* hybridization.

Microdissection: Since a number of our tumors were quite small and had few metastases, but we had to section them to perform PECAM-1 staining, we had intervening frozen sections from these small tumors which we would not be using for the microdissection part of the project. We have utilized these tumor sections for practice microdissection, RNA purification (below), and amplification of cDNA or RNA (below). This practice will enable us to utilize our large, metastatic tumors to best advantage when we finishing analyzing them to find the "hottest spot" and thereby will know which areas to microdissect.

In the interim between the submission of this protocol and the present, Georgetown has acquired a laser microdissection apparatus. The fellow on this project, Dr. Phyllis Vezza, went to the NIH and attended a training session on laser microdissection which was organized by personnel from the laboratory of Dr. Lance Liotta, where laser microdissection was developed. Dr. Liotta's group has shown that frozen sections give the best yield of high-quality RNA (when compared to paraffin sections) (11) and others have determined that hematoxylin seems to have deleterious effects on nucleic acids isolated from microdissected materials (12). However, the effect of immunohistochemistry procedures on RNA quality is unknown. Since we are interested in microdissecting areas of tumors from microvessel hot spots but wish to avoid harvesting areas containing high proportions of mouse cells, we would like to be able to microdissect from PECAM-1 stained sections counterstained to reveal other tissue morphology. We are in the process of determining the best methods for intact RNA recovery. Currently, our procedure consists of subjecting the frozen sections to PECAM-1 immunohistochemistry as above, except that eosin is substituted for hematoxylin as a counterstain. The section is then dehydrated through graded alcohols and xylenes as above, and allowed to air-dry. This method allows identification of morphologic features and accurate microdissection of an approximately 30 μ m spot of tissue.

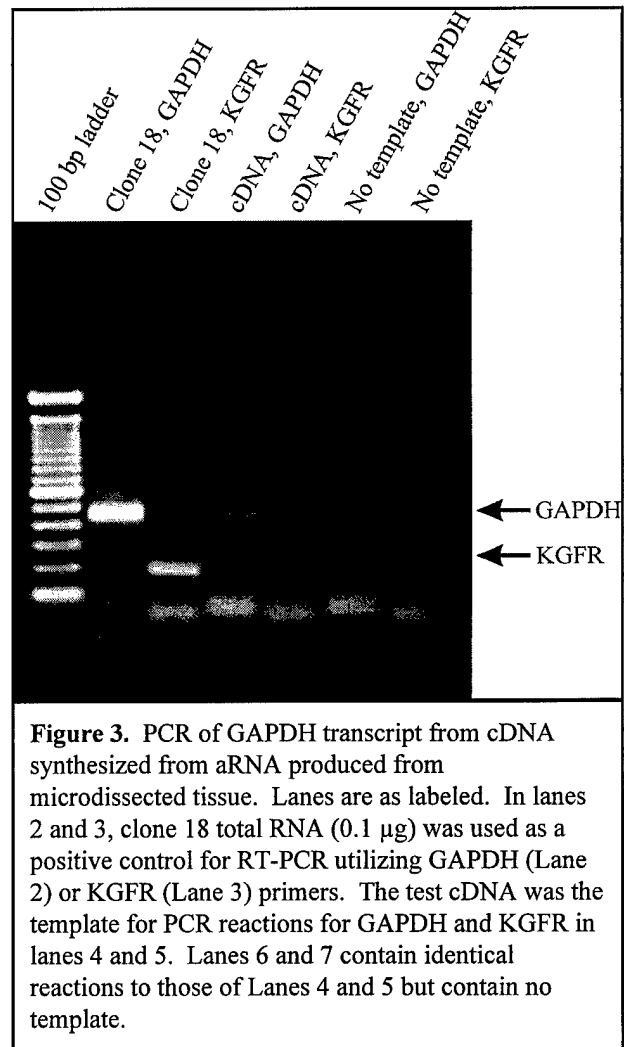
RNA purification: After comparing various methods for RNA purification, we have settled on the Qiagen RNeasy columns (Qiagen, Inc., Santa Clarita, CA). These columns bind RNA longer than 200 bases. This eliminates tRNAs, and small ribosomal RNAs from the preparation, as well as small degraded fragments. Tissue is microdissected directly onto a cap for a microcentrifuge tube. The cap is placed on a tube and an appropriate volume of lysis buffer from the kit is squirted from a tuberculin syringe with the needle inserted through the bottom of the tube. The buffer is aspirated back into the syringe and repeatedly squirted against the cap, insuring that all the microdissected tissue is dislodged from the cap and sheared through the 25 gauge syringe needle. The sample is then applied to the column per manufacturer's directions and RNA eluted in a volume of 30 μ l of water. Although this kit preferentially isolates RNA, very small amounts of DNA contamination are likely. For that reason, we have treated the isolated RNA with 0.1 unit/ μ l RQ1 DNase (Promega, Madison, WI) before using it for downstream applications (below). The RNA is then precipitated with ethanol and resuspended in an appropriate volume of water (usually about 5 μ l) before proceeding to the next step.

Amplification of cDNA or RNA: Because of the small amount of RNA obtained from the microdissected tissue, before we can subject it to differential cloning techniques (below), we must amplify it somehow so it can be ligated into a cloning vector. Synthesis of cDNA from the isolated messenger RNA (which is about 5% of the total RNA) is followed by amplification procedures. There are two possibilities for amplification. The first involves synthesis of cDNA with

manufactured sequences on each end followed by PCR amplification of the resultant cDNA using PCR primers to the manufactured sequences. The resultant amplified cDNA can be ligated into a cloning vector. This method will be referred to as the Clontech method because we have utilized the SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA) to accomplish it. The second involves synthesis of cDNA with a T7 RNA polymerase promoter sequence at the poly-dT end and a manufactured sequence at the other end. Amplified RNA (aRNA) is then synthesized using T7 RNA polymerase. This amplified RNA can then be used for second round cDNA synthesis using the manufactured sequence and the T7 promoter sequence as primers. ARNA can again be synthesized in still greater quantities, which can be used for a third round of cDNA synthesis, as before, which can be ligated into a cloning vector. This method will be referred to as the aRNA method (13-15).

There are two concerns for any method of amplification. The first is *fidelity*, in that the amplification must preserve relative abundances of particular transcripts in the original mRNA population. The second concern is *yield*, in that each step of purification or ligation has losses, so that a method with numerous steps or steps which have high losses will produce a library which does not contain adequate numbers of individual cDNA species and thus will not be representative of the original population. Each of the methods as outlined above has its particular problems; the Clontech method's problems are mainly fidelity ones and the aRNA method's problems are mainly yield ones. However, since we are starting with very small amounts of RNA, yield is a problem with either method.

Although we have not made a firm decision about which method to use, we are leaning



toward the aRNA method. This is because the amplification achieved by synthesizing RNA from the T7 promoter is arithmetic as opposed to the exponential amplification of PCR in the Clontech method. We are concerned that PCR amplification, even using the same primers for all cDNA species, will amplify certain species preferentially, skewing the abundance of particular species in the final product. Since we wish to compare transcript abundances in different RNA populations in our cloning method, it is essential that we preserve the relative abundances of particular species as represented in the population. We are using both the Clontech and aRNA techniques in connection with another project in the laboratory, which also starts out with small amounts of RNA. Our experience in this other project will enable us to make an intelligent choice concerning these methods.

Utilizing the aRNA method, we have been able to make third-round cDNA and detect the cDNA for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Briefly, RNA from microdissected tissue purified with the RNeasy method was reverse transcribed with Superscript II (Life Technologies, Gaithersburg, MD) using a poly-dT-T7 primer as described (13). The 3' end of the first strand was tagged with a manufactured sequence through the technique of template switching (16). Second strand cDNA synthesis was performed utilizing a primer to the manufactured sequence and a combination of Klenow fragment and T4 DNA polymerase. The resulting double-stranded cDNA was polished and utilized as template for aRNA synthesis by T7 RNA polymerase (13). Second round cDNA synthesis was performed utilizing the aRNA as template and a primer to the manufactured sequence for the first strand and the poly-dT-T7 primer for the second strand. Second round aRNA was transcribed from this second round cDNA as from the first round cDNA. Third round cDNA synthesis was accomplished in the same way as the second round using the second round aRNA as template. The cDNA resulting from this synthesis was used in a PCR reaction with primers for human GAPDH (Figure 3, lane 4). We were not able to detect the much less abundant transcript for the human keratinocyte growth factor receptor (KGFR) at this level of amplification (positive control is in lane 3 of Figure 3, experimental sample without a KGFR band is lane 5).

Detection of differential gene expression: As mentioned, we are currently involved in another project in the laboratory which involves detection of differential gene expression in 8 RNA populations. This project will have approximately 15 RNA populations to compare (microdissected spots from areas of high microvessel density from the 5 most metastatic tumors to be compared with microdissected spots from two other areas of each tumor not involved with microvessel hot spots). Because of the high likelihood of false positive results with differential display PCR and the labor-intensive necessity of screening all candidate fragments to confirm their differential gene expression, we have been investigating alternative ways of screening for differentially expressed genes. We

McLeskey, Sandra W.
DAMD17-94-J4173

have identified a technique known as amplified fragment-length polymorphism (AFLP) which was first used to detect DNA polymorphisms, but can be used on cDNA to show differences in gene expression. This method makes use of manufactured sequences on the ends of the cDNA which serve as the basis for PCR primers which are degenerate in only the 3' two bases, making the PCR reaction much cleaner and more specific than that of differential display PCR, which uses random decamers as primers. We plan to use this technique on this project also. The particulars of this method will be included in next year's report.

Conclusion

Scientific conclusions to date:

1. Confirming previous results, degree of pulmonary micrometastasis in the present study is tightly correlated with tumor size.
2. We have developed a method of immunostaining followed by microdissection, RNA purification, amplification of mRNA, and cDNA synthesis which produces quantities of cDNA sufficient for PCR of a GAPDH fragment. Refinements to this protocol will enable us to obtain sufficient cDNA from microdissected samples to perform analyses of differential gene expression.

Discussion: This research is by definition risky. It is possible that we will go through the whole procedure and obtain completely negative results. The degree of metastasis may not correlate with microvessel density at areas of microvessel "hot spots". There may not be differences in gene expression which are unique to areas of tumor adjacent to microvessel hot spots. However, the techniques we are developing are extremely powerful ones. We are currently exploring ways of examining gene expression in single endothelial cells in human tumors. The techniques of microdissection, RNA purification, etc., which we are developing in connection with this project will help us with that one. Therefore, even if the scientific conclusions in this project are negative, the methodologic advances will be considerable, and may pave the way for increased knowledge of the process of angiogenesis in human breast cancer.

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McLeskey, Sandra W.
DAMD17-94-J4173

APPENDIX

LIST OF ABBREVIATIONS AND ACRONYMS

ABC	Avidin-biotin complex reagent
AFLP	Amplified fragment-length polymorphisms
a.k.a.	Also known as
BrdU	Bromodeoxyuridine, a thymidine analog
cDNA	Copy DNA
Clone 18	A clonal cell line of ML-20 cells (below) transfected with FGF-1
DNA	Deoxyribosenucleic acid
FGF	Fibroblast growth factor
FGF-1	Fibroblast growth factor 1, a.k.a. acidic FGF
FGF-4	Fibroblast growth factor 4, a.k.a. Kaposi FGF, <i>hst-1</i>
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
KGFR	Keratinocyte growth factor receptor
<i>lacZ</i>	A bacterial gene encoding β -galactosidase
MCF-7	An estrogen receptor positive breast carcinoma cell line
ML-20	A clonal cell line of MCF-7 cells transfected with <i>lacZ</i>
MMVD	Microvessel density
PCR	Polymerase chain reaction
PECAM-1	Platelet-endothelial cell adhesion molecule 1
PBS	Phosphate buffered saline
RNA	Ribosenucleic acid
RT-PCR	Reverse transcription followed by the polymerase chain reaction
T4	Bacteriophage T4
T7	Bacteriophage T7
X-gal	5-bromo-4-chloro-3-indoyl- β -galactoside

McLeskey, Sandra W.
DAMD17-94-J4173

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Publications since the last report: None in connection with this project.

Manuscripts in preparation: None to date.

Personnel receiving pay from this negotiated effort during this period:

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